Deletion of the Kinase Domain in Death-Associated Protein Kinase Attenuates Tubular Cell Apoptosis in Renal Ischemia-Reperfusion Injury

MASANORI KISHINO,* KAZUNORI YUKAWA,† KATSUAKI HOSHI KIMURA,* AKIHOKO SHIRASAWA,* TETSUJIN TANAKA,§ KYOKO OWADA-MAKABE,† YUJI TSUBOTA,† MASANOBU MAEDA,† MASAKAZU INOSE,* KIYOSHI TAKEDA,# SHIZUO AKIRA,# and MASATOSHI MUNE*

Departments of *Internal Medicine, †Physiology, ‡Legal Medicine, §Anatomy, and ¶Gynecology and Obstetrics, Wakayama Medical University, Wakayama, Japan; and #Department of Host Defense, Research Institute for Microbial Disease, Osaka University, Osaka, Japan.

Abstract. Death-associated protein kinase (DAPK) is a calcium/calmodulin-dependent serine/threonine kinase localized to renal tubular epithelial cells. To elucidate the contribution of DAPK activity to apoptosis in renal ischemia-reperfusion (IR) injury, wild-type (WT) mice and DAPK-mutant mice, which express a DAPK deletion mutant that lacks a portion of the kinase domain, were subjected to renal pedicle clamping and reperfusion. After IR, DAPK activity was elevated in WT kidneys but not in mutant kidneys (1785.7 ± 54.1 pmol/min/mg versus 160.7 ± 60.6 pmol/min/mg). Furthermore, there were more TUNEL-positive nuclei and activated caspase 3–positive cells in WT kidneys than in mutant kidneys after IR (24.0 ± 5.9 nuclei or 9.4 ± 0.6 cells per high-power field [HPF] versus 6.3 ± 2.2 nuclei or 4.4 ± 0.7 cells/HPF at 40 h after ischemia). In addition, the increase in p53-positive tubule cells after IR was greater in WT kidney than in mutant kidneys (9.9 ± 1.4 cells/HPF versus 0.8 ± 0.4 cells/HPF), which is consistent with the theory that DAPK activity stabilizes p53 protein. Finally, serum creatinine levels after IR were higher in WT mice than in mutant mice (2.54 ± 0.34 mg/dl versus 0.87 ± 0.24 mg/dl at 40 h after ischemia). Thus, these results indicate that deletion of the kinase domain from DAPK molecule can attenuate tubular cell apoptosis and renal dysfunction after IR injury.

Received July 2, 2003. Accepted April 19, 2004.
Masanori Kishino, Kazunori Yukawa, and Katsuaki Hoshino contributed equally to this work.

Correspondence to Dr. Masatoshi Mune, Department of Internal Medicine, Wakayama Medical University, 811-1 Kimiidera, Wakayama 641-8509, Japan. Phone: 81-73-441-0619; Fax: 81-73-441-2877; E-mail: mmune@wakayama-med.ac.jp
1046-6678/1507-1826
Journal of the American Society of Nephrology
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DOI: 10.1097/01.ASN.0000131527.59781.F2

Ischemic acute renal failure (ARF) represents an important clinical problem with high mortality rates (1). Histochemical and molecular study of renal ischemia-reperfusion (IR) (2–4) has characterized several factors associated with renal IR injuries, including reactive oxygen species (5), nitric oxide (6–8), cell surface molecules (8–12), transcription factor NF-κB (13), growth factors (14), cytokines (15–18), and phospholipid messengers (8,19,20). These inflammatory components induce tubule cell necrosis or apoptosis and result in ARF (21,22). Mild or short-duration ischemia results primarily in apoptosis rather than necrosis (2,23). This represents a mechanism that could potentially be interrupted to prevent ARF (24). Thus, characterization of the molecular mechanisms underlying apoptosis in this case may be of therapeutic benefit.

Intracellular mediators, such as calpain and p53, have been implicated in renal cell apoptosis in IR injuries (25,26). Furthermore, the mitogen-activated protein family of kinases, such as JNK and p38, likely play crucial roles in the response of renal tubule cells to ischemic and toxic stresses associated with ARF (3,24). However, the exact mechanism by which these kinases mediate apoptosis of renal tubule cells in ischemia-induced ARF remains unclear (3,24).

Death-associated protein kinase (DAPK) is a calcium/calmodulin-dependent kinase that phosphorylates serine/threonine residues on proteins (27,28). DAPK modulates cell death induced by IFN-γ (27), TNF-α (29), Fas (29), and detachment from the extracellular matrix (30). DAPK has been also implicated in TGF-β-induced apoptosis in several cultured cell lines in which Smad proteins mediate transcriptional activation of DAPK (31). Because TGF-β1 is a crucial participant in tubular cell apoptosis in chronic progressive kidney disease (32), DAPK may also play a role in injury after renal IR. Indeed, our immunohistochemical analyses have confirmed the localization of DAPK to renal tubular epithelial cells in human and rodents (33). The goal of the study presented here was to investigate whether DAPK activity contributes to renal cell apoptosis in renal IR injury.
Materials and Methods

Animals (Generation of DAPK-Mutant Mice)

Mice expressing a mutant DAPK lacking a portion of the kinase domain (amino acids 22 to 95) were generated by gene targeting in E14.1 embryonic stem (ES) cells (33). Briefly, a targeting vector was designed to replace the exon encoding amino acids 22 to 95 of mature DAPK with the neomycin resistance gene. The vector was introduced into E14.1 ES cells by electroporation. The clones resistant to G418 and ganciclovir were screened by PCR and confirmed by Southern blot analysis. The mutant ES cells were injected into blastocysts (C57BL/6) and transferred to the uteri of pseudopregnant mice to generate chimeras. Chimeras were bred to C57BL/6 mice for germ-line transmission of the mutant allele. Pairs of the resulting heterozygous mice were subsequently bred to gain homozygous DAPK-mutant mice. Wild-type (WT) littermates were used as controls. Animals were housed in the animal facilities of Wakayama Medical University. Our institutional Animal Ethics Review Committee approved all experimental protocols.

Reverse Transcription (RT)-PCR

Total RNA was prepared from kidney tissue with the SV Total RNA Isolation System (Promega, Madison, WI). Total RNA from kidney tissues was transcribed by reverse transcriptase with random primers to make template cDNA. The PCR primers were: 5'-CACATGCCGTGTTGAGCAAGGA-3' (sense primer) and 5'-GATCAGGGGTGCTGCAAAC-3' (antisense primer 1) or 5'-GACGTCATAGCCTGACAAAAGGA-3' (antisense primer 2). Amplification by PCR with KOD-Plus-DNA polymerase (Toyobo, Japan) was performed in 25-μl reaction mixtures with a GeneAmp PCR System thermocycler (Perkin-Elmer Applied Biosystems, Foster City, CA). An initial step was performed at 94°C for 2 min. The PCR cycles were as follows: denaturation at 94°C for 15 s, annealing at 57°C for 30 s, and extension at 68°C for 1 min 15 s for 30 cycles, followed by a final extension step at 68°C for 7 min.

Western Blotting

Identical amounts of proteins quantified by a Coomassie blue assay were loaded in the lanes of a 7.5% Tris-HCl gel, resolved by electrophoresis, and then transferred to a polyvinylidene fluoride filter membrane. DAPK was detected with the ECL Western blotting system (Amersham Pharmacia Biotech, Buckinghamshire, UK) with an anti-DAPK antibody (Sigma, St. Louis, MO).

IR Model (Induction of IR)

Under anesthesia with pentobarbital sodium (50 mg/kg), an abdominal incision was made. Ischemia was induced by clamping the left renal pedicle for 30 min with a vascular clamp (Roboz, Gaithersburg, MD) while mice were kept at constant temperature (37°C) and well hydrated. Then the clamp was removed, and the kidney was allowed to perfuse. At 16, 40, and 120 h after ischemia, animals were anesthetized, and the renal tissues were removed.

To assess the effect of IR on renal function, surgery was performed in the same manner, except for clamping of both renal pedicles. At 40 h after ischemia, mice were killed, and serum and kidney samples were obtained. Sham operation was performed in both WT and mutant mice by manipulation of the renal pedicles without clamping. The sham-operated kidneys were used as controls in each animal. Five animals from each respective genotype were used for each experiment.

DAPK Kinase Assay

Soluble homogenates from mouse kidney regions were prepared by dousing tissue in T-PER Tissue Protein Extraction Reagent (Pierce, Rockford, IL) containing protease inhibitors, α-Complete (Roche, Penzberg, Germany). WT DAPK and mutant DAPK molecules were immunoprecipitated from tissue extracts with Seize X Protein G Immunoprecipitation Kit (Pierce). Initially, anti-DAPK monoclonal antibodies (Sigma) bound to ImmunoPure Immobilized Protein G were cross-linked with disuccinimidyl suberate. Soluble homogenates were mixed and incubated with the antibody-coupled gel at 4°C for 3 h. Then, immunoprecipitated DAPK molecules were eluted, assessed by Western blotting, and quantified. The Coomassie Plus assay (Pierce) was used to assure equal amounts of DAPK in the kinase assay.

The kinase activities of DAPK and mutant DAPK were quantified essentially as described by others (34), with some modifications. The DAPK molecules eluted from the gel were incubated with 200 μM ATP and [γ32P]ATP (2.5 μCi per reaction) in assay buffer (20 mM HEPEs pH 7.5, 1 mM DTT, 2 mM MgCl2, 2 mM MnCl2, 75 mM NaCl, 1 mM sodium orthovanadate, 1 mM okadaic acid, 1× α-Complete) for 10 min at 25°C, either with the biotinylated peptide substrate biotin-(C)6-KKRQPQRRYSNVF (20 μM), or without peptide substrate for determination of background phosphorylation. All reactions were terminated by adding guanidine hydrochloride solution (2.5 M), and samples were spotted onto SAM2® Biotin Capture Membrane (Promega, Madison, WI). The SAM2® Biotin Capture Membrane was washed once for 30 s with 2 M NaCl, then three times for 2 min each with 2 M NaCl, then four times for 2 min each with 2 M NaCl in 1% H3PO4, and then twice for 30 s each with deionized water. Phosphate transfer was measured by scintillation counting.

Tissue Collection and Morphologic Analysis

The kidneys were fixed in 4% buffered paraformaldehyde (pH 7.4). Specimens were then cut into 4-μm thin sections and stained with periodic acid–Schiff.

Assessment of Renal Function

Serum BUN and creatinine levels were measured as markers of renal function with Fujifilm DRI-CHEM 3500V autoanalyzer (Fuji Photo Film, Tokyo, Japan).

Apoptosis Assays (TUNEL)

Apoptotic cells in kidney tissues on slides were visualized with the In situ Apoptosis Detection Kit (Takara Bio, Kyoto, Japan) following the protocol provided by the manufacturer. Initially, paraffin-embedded tissue sections were hydrated. To permeabilize the sections by enzyme solution efficiently, tissues were treated with a buffer containing proteinase K (400 μg/ml) for 5 min at room temperature. After washing with PBS, endogenous peroxidase was blocked by incubation of slides in a solution of 3% H2O2 in water at room temperature for 5 min. After washing with PBS, tissues were then incubated with a reaction buffer containing terminal deoxynucleotidyl transferase (TdT) and FITC-labeled substrate for 90 min at 37°C. Tissues were further incubated with anti-FITC HRP conjugate (Takara Bio) for 30 min at 37°C and reacted with diaminobenzidine solution for 10 min at room temperature to visualize positive nuclei. After counterstaining with hematoxylin, cells on slides were examined by light microscopy.

Immunohistochemical Analysis (Detection of Activated Caspase 3 and p53)

To directly detect activated caspase 3 or p53 in IR kidneys, tissue sections were treated in 10 mM sodium citrate buffer (pH 6.0) and
placed in a microwave oven for antigen unmasking. After blocking with 5% goat serum in PBS, tissue sections were incubated with cleaved caspase 3 (Asp175) antibody (Cell Signaling Technology, Beverly, MA) or with p53 antibody (Cell Signaling Technology) at 4°C overnight. Sections were then incubated with dextran polymer conjugated with secondary antibodies to rabbit Ig and peroxidase (DakoCytomation, Kyoto, Japan), and activated caspase 3 or p53 was visualized in situ by the diaminobenzidine detection procedure.

**Assessment of Immunohistochemical Characteristics and Statistical Analyses**

Over 20 pictures from three immunostained sections per each kidney were taken at a magnification of 400× (high-power field [HPF]) by a light microscope equipped with a 3CCD camera (HV-C20S; Nikon, Tokyo, Japan) and stored in a Macintosh computer connected to a 3CCD camera. The pictures were then analyzed by counting the number of TUNEL-positive nuclei and cleaved caspase 3 or p53 positive cells in a blinded manner by two independent investigators. Data are expressed as mean values ± SE. Comparison between experimental groups was performed by ANOVA followed by the Scheffé test for post hoc analysis. A P value of less than 0.05 was considered statistically significant.

**Results**

**DAPK-Mutant Mice**

Analysis of mRNA expressed in mutant kidneys demonstrated transcripts that encode a mutant DAPK protein lacking amino acids 22 to 95 of the kinase domain (Figure 1, A and B). Western blotting analysis of tissue from mutant mice demonstrated the mutant DAPK with a smaller molecular weight (Figure 1C). BUN and creatinine levels were similar when comparing WT mice to mutant mice. (BUN, 27.88 ± 1.28 mg/dl versus 29.43 ± 3.64 mg/dl; creatinine, 0.48 ± 0.05 mg/dl versus 0.45 ± 0.03 mg/dl).

**Kinase Activity of DAPK during Renal IR**

To investigate the effect of IR on DAPK activity, WT and mutant DAPK was isolated from experimental and sham-operated kidney from all animals at various time points. In WT kidneys, DAPK activity was significantly increased and peaked at 16 h after ischemia, indicating that IR stimuli enhanced DAPK catalytic activity. In contrast, DAPK activity was not significantly increased in mutant kidneys after IR (Figure 2).

**Histologic Evaluation of Kidneys with IR Injury**

Extensive tubular injuries with obstructing granular cast formation and epithelial cell sloughing was present in WT kidneys at 40 h after ischemia (Figure 3), which is consistent with previous reports (2,6–12,14,15). The mutant kidneys after IR displayed similar histopathology, as represented by tubular damage associated with debris and cast formation (Figure 3). However, small condensed and fragmented nuclei representing apoptosis were more prominent in WT kidneys as compared with mutant kidneys (Figure 3, C and D).

**Significant Attenuation of IR-induced Apoptosis in Mutant Kidneys**

To assess the direct contribution of DAPK activity to renal cell apoptosis in IR injuries, TUNEL assays were performed on kidneys from both genotypes at 16, 40, and 120 h after reperfusion. WT kidneys after IR showed an extensive amount of TUNEL-positive staining (Figure 4A, a and c). In contrast, mutant kidneys after IR had fewer TUNEL-positive cells (Figure 4A, b and d). Thus, renal cell apoptosis at 16 and 40 h after ischemia was increased in WT kidneys when compared with mutant kidneys (3.8 ± 1.2 versus 0.5 ± 0.1 nuclei/HPF at 16 h; 24.0 ± 5.9 versus 6.3 ± 2.2 nuclei/HPF at 40 h; Figure 4B).
To further examine the extent of apoptosis after IR, immunohistochemical analyses were performed on kidneys from both genotypes with antibodies detecting endogenous levels of activated caspase 3 (Figure 5). As shown in Figure 5B, the cleaved caspase 3 immunoreactivity was significantly increased in renal tubule cells in postischemic WT kidneys. Although renal tubule cells with activated caspase 3 was increased in mutant IR kidneys, the intensity was still greater in postischemic WT kidneys than in mutant kidneys (3.7 ± 0.6 versus 0.5 ± 0.1 cells/HPF at 16 h; 9.4 ± 0.6 versus 4.4 ± 0.7 cells/HPF at 40 h; 1.4 ± 0.2 versus 0.6 ± 0.1 cells/HPF at 120 h).

Detection of p53 in Renal Tubule Cells after IR
After IR, the amount of tubules cells expressing immunoreactive p53 was increased in WT kidneys when compared with mutant kidneys (9.9 ± 1.4 versus 0.8 ± 0.4 cells/HPF) (Figures 6 and 7).

Effects of the Deletion of DAPK Activity on Renal Function
To examine if the reduction in apoptosis in mutant kidneys had any improvement on renal function after IR injuries, serum creatinine levels were measured in wild and mutant mice at 40 h after IR. After IR, mean creatinine was significantly higher in WT mice than in mutant mice (2.54 ± 0.34 versus 0.87 ± 0.24 mg/dl) (Figure 8).

Discussion
The major findings of this study were (1) that deletion of the kinase domain of DAPK by gene targeting technology successfully inhibited the activation of DAPK in response to renal IR, and (2) that the inactivation of DAPK catalytic activity was associated with reduced apoptosis and protected renal function in response to IR. These data indicate that DAPK kinase activity plays a critical role in apoptosis and development of ARF after IR injury.

Studies have demonstrated that DAPK activity mediates apoptotic cell death of cultured cells in vitro (27–30,31,35,36).
and that primary cultured cells from DAPK null knockout mice have some resistance to apoptotic stimuli (35,37). Furthermore, variants of DAPK may also prevent apoptosis in mice (38). However, a recent experiment measuring the catalytic activity of DAPK in an animal model of brain hypoxic-ischemic injury suggested that DAPK may play a role in neuronal development or recovery from injury rather than directly in apoptosis (39). Thus, aside from its role in cancer (40–43), there are no studies that demonstrate that DAPK play a direct role in the modulation of apoptosis.

In the study presented here, kinase domain mutant DAPK molecules were not activated in response to IR stimuli. This is consistent with previous findings that DAPK catalytic activity is dependent on the kinase domain (27,28,30,35,36). Furthermore, the domain deleted from mutant DAPK protein contains a conserved lysine-42 essential for DAPK catalytic activity.
(28), reinforcing the importance of the lysine residue for full activation of DAPK activity in IR kidneys in vivo. Thus, the mutant mice used in the study presented here serve as good experimental models to characterize the role of DAPK catalytic activity in the apoptotic process in vivo.

In the study presented here, apoptosis was assessed by the TUNEL method and by immunohistochemical detection of activated caspase 3 and p53. Each apoptosis parameter displayed a similar pattern in renal IR peaking at 40 h after ischemia, although TUNEL counts exceeded other parameters, possibly because of the inclusion of necrotic cells. In contrast, DAPK catalytic activity peaked at 16 h after ischemia, although rapidly declined thereafter. The activation pattern of DAPK preceding apoptosis may indicate that DAPK is an integrator of signaling to a pro-apoptosis executioner, such as caspase 3, in the apoptosis pathway (29,35). Indeed, the function of DAPK downstream from death receptors (16,29) may explain account for the early activation of DAPK in response to IR. In fact, the activation pattern of DAPK in renal IR is reminiscent of changes of DAPK activities in ceramide-induced apoptosis of PC12 cells (44). DAPK catalytic activities were transiently increased 10 min after exposure of ceramide and decreased at 90 min, with apoptosis observed at 12 h. Thus, the activation pattern of DAPK may explain why there is a significant difference in kinase activity between genotypes only in postischemic kidneys.

The apoptosis cell number in mutant kidneys after IR was significantly less than that in WT kidneys after IR (Figures 4 and 5), indicating that DAPK catalytic activity mediates at least a portion of apoptosis seen in renal IR injuries. DAPK has been shown to modulate apoptosis induced by IFN-γ, TNF-α, Fas, cellular detachment from extracellular matrix, and ceramide (27,29,30,37). Among these stimuli, TNF-α has been implicated in renal IR injuries (16–18). Thus, renal...

Figure 5. Activated caspase 3 during renal ischemia-reperfusion (IR). (A) Renal tubule cells with the cleaved caspase 3 immunoreactivity were detected in wild-type (WT) kidneys 40 h after IR (WT/IRI). In contrast, there were much fewer renal tubule cells with activated caspase 3 in death-associated protein kinase (DAPK)-mutant kidneys at 40 h after ischemia (MT/IRI). (B) Renal tubule cells with activated caspase 3 were significantly increased in postischemic WT kidneys. Although renal tubule cells with cleaved caspase 3 immunoreactivity were increased in mutant kidneys after IR, the increase was significantly less than that of WT kidneys after IR (*)
IR may include TNF-α, leading to activation of DAPK catalytic activity via the TNF receptor 1 and resulting in renal cell apoptosis. However, IR-induced apoptosis in mutant kidneys was not completely inhibited, which implies that other kinases, such as the mitogen-activated protein kinases family and DAPK family members, are also involved in this process (45–48).

Experiments that used DAPK-deficient rodent embryonic fibroblasts in culture showed that DAPK activity results in...
stabilization of p53 protein in the context of a stress signal (35,49). The p53 protein is an important mediator of renal cell apoptosis after IR, and p53 inhibitors prevent renal cell apoptosis and renal dysfunction (26). In the study presented here, there were significantly fewer renal tubule cells with p53 signal in DAPK-mutant kidneys after IR when compared with WT kidneys after IR (Figures 7 and 8), which is consistent with previous reports showing stabilization of p53 by DAPK in vitro (35). Thus, IR may result in apoptosis via a cascade involving TNF-α, DAPK activation, p53 stabilization, and p53-mediated transcription of apoptosis-inducing genes (49).

The degree of renal dysfunction after IR correlates with the extent of apoptosis (3,22,24,26,50). In the study presented here, serum creatinine levels after IR were lower in DAPK-mutant mice than in WT mice (Figure 8). This is consistent with the principle that inhibition of IR-induced renal cell apoptosis prevents renal dysfunction. Furthermore, these results suggest that agents that modulate DAPK activity are attractive candidates to produce clinical therapeutic benefits in patients with acute ischemic renal failure.

Acknowledgment
We thank Chiaki Yamashita for her technical assistance.

References


